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(54) Title: METHODS FOR THE TREATMENT OF INFLAMMATORY JOINT DISEASE

(57) Abstract

The use of compounds that block complement component C5 or its active fragments C5a and/or C5b (such compounds collectively referred to as "C5 blockers") to treat established joint inflammation (arthritis) is disclosed. Administration of such C5 blockers has been found to: 1) arrest and/or reduce inflammation in joints which are already inflamed, and 2) inhibit the spread of inflammation to unaffected joints.

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5 METHODS FOR THE TREATMENT OF INFLAMMATORY JOINT DISEASEFIELD OF THE INVENTION

The present invention relates to methods for the treatment of inflammatory joint disease. In particular, 10 the invention relates to the use of blockers of complement component C5 ("C5 blockers") as pharmaceutical agents to treat established joint inflammation.

BACKGROUND OF THE INVENTIONI. The Complement System

15 The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane 20 cofactors. The plasma proteins (which are also found in most other body fluids, such as lymph, bone marrow, synovial fluid, and cerebrospinal fluid) make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by 25 interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

30 The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and, while they differ in their early steps, both converge and share the same terminal complement components responsible for the destruction of 35 target cells and viruses.

The classical complement pathway is typically initiated by antibody recognition of and binding to an

antigenic site on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1. The C1 thus bound undergoes a set of autocatalytic reactions that result in, *inter alia*, the induction of C1 proteolytic activity acting on complement components C2 and C4.

This activated C1 cleaves C2 and C4 into C2a, C2b, C4a, and C4b. The function of C2b is poorly understood. C2a and C4b combine to form the C4b,2a complex, which is an active protease known as classical C3 convertase. C4b,2a acts to cleave C3 into C3a and C3b. C3a and C4a are both relatively weak anaphylatoxins that may induce degranulation of mast cells, resulting in the release of histamine and other mediators of inflammation.

C3b has multiple functions. As opsonin, it binds to bacteria, viruses and other cells and particles and tags them for removal from the circulation. C3b can also form a complex with C4b,C2a to produce C4b,2a,3b, or classical C5 convertase, which cleaves C5 into C5a (another anaphylatoxin), and C5b. Alternative C5 convertase is C3b, Bb, C3b and performs the same function. C5b combines with C6 yielding C5b,6, and this complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of a cell membrane. Upon binding of C9, the complete membrane attack complex (MAC) is formed (C5b-9) which mediates the lysis of foreign cells, microorganisms, and viruses.

Further discussions of the classical complement pathway, as well as a detailed description of the alternative pathway of complement activation, can be found in numerous publications including, for example, Roitt, et al., 1988, and Muller-Eberhard, 1988.

II. Joint Inflammation

A variety of common medical disorders have as a common element the inflammation of the patient's joints. In the United States alone, millions of patients suffer from such joint inflammation. Afflicted individuals are

frequently disabled, and the costs of medical care for patients suffering from such disorders are significant. While numerous means are available for treatment of joint inflammation, and new treatments continue to become 5 available, none of these is as safe and effective as could be desired, and there has thus been a long felt need for new approaches and better methods to control joint inflammation.

Clinically, joint inflammation is associated with 10 joint stiffness, pain, weakness, and sometimes joint fatigue. Uniformly, the joint is tender and swollen, and often erythematous. Diagnosis of the inflammatory nature of the joint disease is frequently based upon this typical clinical presentation as well as upon 15 radiographic examination and aspiration and examination of synovial joint fluid. Examination of joint fluid of an inflamed joint generally reveals elevation of various markers of inflammation, such as, leukocytes (including neutrophils), antibodies, cytokines, cell adhesion 20 molecules, and complement activation products (De Clerck et al., 1989; Heinz et al., 1989; Moffat et al., 1989; Peake et al., 1989; Brodeur et al., 1991; Firestein et al., 1991; Matsubara et al., 1991; Olsen et al., 1991; Oleesky et al., 1991; Jose et al., 1990; Zvaifler, 1968; 25 Zvaifler, 1969a; Zvaifler, 1969b; Zvaifler, 1974; Ward and Zvaifler, 1971; Ward, 1975; Moxley and Ruddy, 1985; Mollnes et al., 1986; Auda et al., 1990; Olmez et al., 1991; Kahle et al., 1992; Koch et al., 1994; Thorbecke et al., 1992; Saura et al., 1992; Feldman et al., 1990; 30 Feldman et al., 1991; Fong et al., 1994; Harigi et al., 1993; Morgan et al., 1988; Shingu et al., 1994; Abbink et al., 1992; and Corvetta et al., 1992). Radiographic examination of affected joints generally reveals soft tissue swelling and/or rosive changes.

Joint inflammation is associated with a group of 35 diseases that are referred to medically as arthridities (types of arthritis). The term "arthritis" is used

medically to generally describe diseases of the joints. The term, however, is also used to describe certain medical conditions, of which rheumatoid arthritis (RA) is the primary example, that consist of a multiplicity of different pathologic manifestations, including, but by no means limited to, joint disease.

Discussions of arthritis may thus include diseases such as RA, where joint disorders are only one facet of the varied pathologies associated with the disease. The present invention is directed specifically to the joint disorder aspects of these diseases. The methods of the invention, however, may also have beneficial effects on non-joint-associated pathologies. For example, use of the methods of the invention to treat established joint inflammation associated with RA, psoriasis, lupus, and other disorders may also provide therapeutic benefits impacting on some of the other pathologic manifestations of these multifaceted disease states, such as vascular inflammation and nephritis (see, for example, Wurzner, et al., Complement Inflamm. 8:328-340, 1991, U.S. Application Serial No. 08/217,391 filed on March 23, 1994, U.S. Application Serial No. 08/236,208 filed on May 2, 1994, and Sims, et al., U.S. Patent No. 5,135,916).

It should be noted that the present invention is not concerned with all types of joint disorders, but only those involving inflammation. Thus, for example, the invention is applicable to the treatment of late-stage osteoarthritis (OA), which is an inflammatory joint disease, but generally not to early stage OA, which does not typically have a significant inflammatory component.

Detailed discussions of the arthridities can be found in numerous medical texts, including Arnett, 1992. Cecil Textbook of Medicine, Wyngaarden et al. (eds.), W.B. Saunders Company, Philadelphia, Chapter 258, pp. 1508-1515; Lipsky, 1994. Harrison's Principles of Internal Medicine, 13th Ed., Isselbacher et al. (eds), McGraw-Hill, Inc., New York, Chapter 285, pp. 1648-1655;

and McCarty and Koopman, 1993. Arthritis and Allied Conditions, 12th Ed. Lea and Febiger, Philadelphia. As discussed in detail in these and other texts, and reviewed below, joint inflammation is associated with numerous local and systemic disease processes.

5 Factors Associated with Joint Inflammation

Joint inflammation is a complex process involving, among other things, activation of both cellular and humoral immune responses.

10 Cellular immune responses include infiltration by white blood cells, predominantly neutrophils (also referred to as polymorphonuclear cells or PMNs). Mononuclear white blood cell infiltrates are also common in many inflamed joints. Infiltrating mononuclear cells, including T lymphocytes (as well as cells resident within the joint such as synovial cells, fibroblasts, and endothelial cells) are activated and contribute to the production of multiple inflammatory cytokines including TNF- α , IL-1, IFN- γ , IL-2, IL-6, IL-8, GM-CSF, PDGF and FGF, these latter two being capable of stimulating synovial cell proliferation.

15 20 25 All of these cytokines are thought to play a role in inducing the production of numerous other inflammatory factors as well as various other mediators of tissue degradation. These factors and mediators of degradation include products of arachidonic acid metabolism (that are active in various intracellular signal transduction pathways), reactive oxygen intermediates, and degradative enzymes such as collagenase, stromelysin, and other neutral proteases, all of which can further contribute to the inflammatory response and to tissue destruction.

30 35 Cellular infiltration into the synovium is enhanced by the upregulation of cell adhesion molecules such as selectins, LFA-3, and members of the ICAM family of Ig-like cell adhesion molecules on cells within the joint. These adhesion molecules promote the infiltration of activated white blood cells into the affected joint by

stimulating leukocyte (including lymphocyte) adhesion, migration, and activation.

The humoral immune system also contributes to joint inflammation. Antibodies are produced within inflamed joints in such diseases as RA, JRA and OA (see below) and generate localized immune complexes that can activate the complement system. As discussed above in greater detail, activated complement components can have cytolytic, cell activating, anaphylatoxic, and chemotactic effects.

These multifactorial inflammatory responses lead to cartilage destruction and bone erosion that ultimately result in the joint deformity seen in patients with chronic joint inflammation.

In view of the complex nature of joint inflammation, a variety of theories, many of which are conflicting, have been proposed in the art to explain the relative importance of the various factors involved. Notwithstanding extensive work, there remains a basic controversy in the art as to the relative roles of the cellular and humoral immune systems in joint inflammation, including what role complement plays in such inflammation. See, for example, Andersson and Holmdahl, 1990; Brahn and Trentham, 1989; Chiocchia et al., 1990; Chiocchia et al., 1991; Durie et al., 1993; Goldschmidt et al., 1990; Goldschmidt and Holmdahl, 1991; Holmdahl et al., 1985; Holmdahl et al., 1989; Holmdahl et al., 1990; Hom et al., 1988; Hom et al., 1992; Hom et al., 1993; Mori et al., 1992; Myers et al., 1989A; Nakajima et al., 1993; Osman et al., 1993; Peterman et al., 1993; Seki et al., 1988; Seki et al., 1992; Terato et al., 1992; Watson et al., 1987; and, in particular, the following reports relating to the complement system and/or the relative roles of T cells and complement components in joint inflammation: Andersson et al., 1991; Andersson et al., 1992; Banerjee et al., 1988B; Banerjee et al., 1989; David, 1992; Fava et al., 1993; Haqqi et al., 1989; Kakimoto et al., 1988; Maeurer et

al., 1992; Morgan et al., 1981; Moxley and Ruddy, 1985; Reife et al., 1991; Spinella et al., 1991; Spinella and Stuart, 1992; van Lent et al., 1992; Watson et al., 1987; Watson and Townes, 1985; and Williams et al., 1992A. To date, these wide ranging studies have not led to effective treatments for established joint inflammation based on modulation of the complement system and, in particular, based on the use of C5 blockers.

The studies of the prophylactic effects of C5 blockers reported below in Example 2 were designed to determine if C5 was an appropriate and effective target for pharmacological modulation of the humoral immune system in order to prevent joint inflammation. The surprising effectiveness of C5 blockers in preventing onset of joint inflammation led to the design and execution of the studies reported in Example 1 in which C5 blockers were used to treat established inflammation. At the outset of these experiments, it was anticipated that such treatment would have little measurable effect upon established joint inflammation, as it was supposed that C5 was more important early in the disease process when the chemotactic activity of C5a would trigger the infiltration of inflammatory cells. It was further supposed that the involvement of T cells in established disease would continue to provide significant inflammatory stimuli even in the absence of C5 activity. As shown by the results of Example 1 this expectation was incorrect in that C5 blockers were found to be surprisingly effective in arresting and/or reducing the inflammation of joints which were already inflamed, while at the same time inhibiting the spread of inflammation to unaffected joints.

Diseases Commonly Associated with Joint Inflammation

Rheumatoid arthritis (RA) and juvenile onset rheumatoid arthritis (JRA) are chronic multisystem diseases of unknown cause. RA affects approximately 1% of the population, with women affected three times more

commonly than men. The onset is most frequent during the fourth and fifth decades of life. RA and JRA are systemic diseases with numerous pathologic manifestations in addition to their joint inflammatory aspects. In RA, 5 these manifestations include RA vasculitis (inflammation of the blood vessels), which can affect nearly any organ system and can cause numerous pathologic sequelae including polyneuropathy, cutaneous ulceration, and visceral infarction. Pleuropulmonary manifestations 10 include pleuritis, interstitial fibrosis, pleuropulmonary nodules, pneumonitis, and arteritis. Other manifestations include the development of inflammatory rheumatoid nodules on a variety of periarticular structures such as extensor surfaces, as well as on 15 pleura and meninges. Weakness and atrophy of skeletal muscle are common.

The joint inflammation aspects of RA present as persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. In 20 general, the complex intraarticular inflammatory response seen in RA is of the type described above in the general discussion of joint inflammation.

Many patients with systemic lupus erythematosis (SLE) also develop joint inflammation referred to as 25 lupus arthritis. SLE is an autoimmune disease of unknown cause in which numerous different cells, tissues, and organs are damaged by pathogenic autoantibodies and immune complexes. Clinical manifestations of SLE are numerous and include a variety of maculopapular rashes, 30 nephritis, cerebritis, vasculitis, hematologic abnormalities including cytopenias and coagulopathies, pericarditis, myocarditis, pleurisy, gastrointestinal symptoms, and the aforementioned joint inflammation.

Osteoarthritis (OA) represents the most common joint 35 disease of mankind, and OA of the knee is the leading cause of chronic disability in developed countries. It is manifested by pain, stiffness, and swelling of the

involved joints. Articular cartilage, responsible for the most critical mechanical functions of the joint, is the major target tissue of OA. The breakdown of articular cartilage in OA is mediated by various enzymes such as metalloproteinases, plasmin, and cathepsin, which are in turn stimulated by various factors that can also act as inflammatory mediators. These factors include cytokines such as interleukin-1, which is known to activate the pathogenic cartilage and synovial proteases.

The identification of above normal levels of immunoglobulins in cartilage in generalized OA and the demonstration of type II collagen-specific antibodies in some OA patients provide evidence of a role for immune activation in this disease state (see, for example, Jaslin, 1989). The observation that OA rarely remains monoarticular also suggests that this disease is a systemic disorder of articular cartilage. Synovial inflammation becomes more frequent as the disease progresses. In fact, in late stage OA, histologic evidence of synovial inflammation may be as marked as that in the synovium of patients with RA-associated joint inflammation.

Psoriatic arthritis is a chronic inflammatory joint disorder that affects 5 to 8% of people with psoriasis. A significant percentage of these individuals (one-fourth) develop progressive destructive disease. Twenty-five percent of psoriasis patients with joint inflammation develop symmetric joint inflammation resembling the joint inflammation manifestations of RA, and over half of these go on to develop varying degrees of joint destruction.

Other Diseases Associated with Joint Inflammation

A variety of other systemic illnesses have joint inflammation as a prominent feature of the clinical presentation.

Peripheral joint inflammation occurs in as many as one-fifth of patients with inflammatory bowel disease.

The joint inflammation is acute, associated with flare-ups of the bowel disease, and is manifested by swollen, erythematous, warm, and painful joints. Synovial fluids of sufferers have an acute inflammatory exudate of mostly neutrophils, and radiographs demonstrate soft tissue swelling and effusions.

The synovitis that accompanies hepatitis B resembles serum sickness, with abrupt onset of fever and articular inflammation. There is generally a symmetric inflammation of joints including the knee, shoulder, wrist, ankles, elbow, and the joints of the hands. Immune complexes containing hepatitis B antigens are present in serum and synovium, lending support to the concept that the synovitis is immunologically mediated. Other viral diseases associated with joint inflammation include rubella, human immunodeficiency virus infection, coxsackieviral, and adenoviral infections.

An immune complex mediated joint inflammation is also associated with intestinal bypass surgery, and joint inflammation is a prominent manifestation of Whipple's disease, or intestinal lipodystrophy, where fever, edema, serositis, lymphadenopathy, uveitis, and cerebral inflammation are associated findings. Furthermore, potentially immunologically-related joint inflammation is an associated sequela of infectious endocarditis and certain spirochetal infections, most notably infection with Borrelia burgdorferi, the causative organism of Lyme disease.

Primary Sjögren's syndrome is a chronic, slowly progressive autoimmune disease characterized by lymphocytic infiltration of the exocrine glands resulting in xerostomia and dry eyes. One-third of patients present with systemic manifestations, including vasculitis, nephritis, mononeuritis multiplex, and, most commonly, joint inflammation.

Ankylosing spondylitis (AS) is an inflammatory disorder of unknown cause that affects primarily the

axial skeleton, but peripheral joints are also affected. Its incidence correlates with the HLA-B27 histocompatibility haplotype, and immune-mediated mechanisms are further implicated by elevated serum 5 levels of IgA and an inflammatory joint histology with similar characteristics to those seen in the joint inflammation aspects of RA. Thus, synovial fluid from inflammatory peripheral joints in AS is not distinctly different from that of other inflammatory joint diseases.

10 Reactive arthritis (ReA) refers to acute nonpurulent joint inflammation complicating an infection elsewhere in the body. Reactive arthritis is believed to be immunologically mediated. Included in this category is the constellation of clinical findings often referred to 15 as Reiter's syndrome or Reiter's disease. In addition to joint inflammation, this syndrome affects the skin, eyes, mucous membranes, and less commonly the heart, lungs, and nervous system. Reiter's syndrome may follow enteric infections with any of several Shigella, Salmonella, 20 Yersinia, and Campylobacter species, and genital infections with Chlamydia trachomatis. The histology of joints affected by this syndrome is similar to that seen 25 in other types of joint inflammation. The joint inflammation is usually quite painful, and tense joint effusions are not uncommon, especially in the knee. The joint inflammation is usually asymmetric and additive, with involvement of new joints occurring over a period of a few days to several weeks.

III. Current Therapies

30 Current therapies for the various types of joint inflammation discussed above include the administration of anti-inflammatory drugs such as non-steroidal drugs, including aspirin, and non-specific immunosuppressive drugs, such as gold compounds, corticosteroids, 35 penicillamine, hydroxychloroquine, methotrexate, azathioprine, alkylating agents such as cyclophosphamide, and sulfasalazine. Administration of each of these

agents is sometimes associated with severe side effects and toxicities. Patients receiving certain of these treatments are also exposed to the dangers of opportunistic infection and increased risk of neoplasia
5 associated with generalized immunosuppression. In addition to the medical texts cited above, discussions of drugs used to treat established joint inflammation can be found in Goodman and Gilman's The Pharmacological Basis of Therapeutics 18th Ed., Gilman et al. (eds.) 1990, Pergamon Press, Inc., New York, Chapter 26, pp. 638-681; Physician's Desk Reference 47th Ed., 1993, Medical Economics Co., Inc., Montvale, NJ; The United States Pharmacopeia 22nd Ed., 1989, Mack Printing Co., Easton, PA; Drug Evaluations Annual 1991, 1990, American Medical Association, Milwaukee, WI; and Cash and Klippel, 1994, N. Eng. J. Med. 330, pp. 1368-1375.

In addition to pharmacologic treatments, relief of the symptoms of joint inflammation is sometimes achieved with warm or cold soaks. Surgical intervention using tendon release procedures and/or joint replacement procedures is frequently the last resort for treatment of chronic joint inflammation. Such orthopedic surgery is associated with increased infection and prostheses have limited life spans.
20

New therapeutic approaches currently being developed include attempts to address various elements of the cellular immune response contributing to the inflammatory cascade present in inflamed joints. These approaches include the administration of therapeutic preparations
25 including anti-T cell and/or anti-cytokine agents (see, for example, Banerjee et al., 1988A; Cannon et al., 1990; Chiocchia et al., 1991; Elliot et al., 1993; Fava et al., 1993; Fujimori et al., 1993; Griswold et al., 1988; Hom et al., 1988; Hom et al., 1991; Hom et al., 1993; Inoue et al., 1993; Kakimoto et al., 1992; Kleinau et al., 1989; Myers et al., 1989b; Myers et al., 1993; Nagler-Anderson et al., 1986; Nishikaku and Koga, 1993; Peterman
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et al., 1993; Piguet et al., 1992; Smith et al., 1990; Spannaus-Martin et al., 1990; Thompson et al., 1988; Trentham et al., 1993; Williams et al., 1992b; Williams et al., 1994; and Wolos et al., 1993).

5 SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for treating established joint inflammation.

To achieve this goal, the invention provides methods involving the use of blockers of complement component C5 10 as pharmaceutical agents to accomplish therapeutic treatment of established joint inflammation. The C5 blockers are administered to animals, e.g., humans, having at least one inflamed joint. The blockers can be 15 administered systemically or locally. They achieve a reduction or stabilization of the inflammation of joints that are already inflamed and inhibit the spread of inflammation to unaffected joints.

As used herein, a "C5 blocker" is a compound that 20 directly interacts with C5, C5a, and/or C5b, i.e., a compound that directly binds to or directly modifies (i.e., by a direct chemical reaction) one of these complement components, so as to inhibit the formation of and/or physiologic function of C5a and/or C5b. 25 Preferably, the formation and/or physiologic functions of both C5a and C5b are inhibited by the C5 blocker.

Direct interaction with C5, C5a, and/or C5b has the important advantage that other components of the complement cascade may be left intact. In particular, 30 the opsonization functions associated with the activation of complement component C3 by a C3 convertase to yield C3a and C3b may be left intact allowing for continued clearance of foreign particles and substances from the body by the action of C3b. The most preferred C5 blockers are those which are of this type, i.e., those 35 that do not interfere with C3b function.

As demonstrated by the examples presented below, in accordance with the invention, it has been surprisingly found that treatment with C5 blockers will arrest and, in many cases, at least partially reverse the disease process at an inflamed joint, while at the same time preventing progression of joint inflammation to non-affected joints. Given the prior understanding in the art regarding the role of the cellular immune system in joint inflammation (see above), one would not have expected that a C5 blocker would have such a dramatic beneficial effect on established joint inflammation.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-c are photomicrographs of joints stained with Hematoxylin and Eosin illustrating the use of a C5 blocker to stop the progression of established joint inflammation. Figure 1a shows a paw joint from a normal mouse; Figure 1b shows an initially affected paw joint from a control treated mouse, where the joint exhibits extensive bone erosion with severe inflammatory cell infiltration and thickening of the synovial membrane; and Figure 1c shows an initially affected paw joint from a C5 blocker treated mouse showing preserved joint structure with some degree of synovial membrane thickening and a surprising lack of polymorphonuclear cell infiltration compared with the initially affected joint from the control group (Figure 1b).

Figures 2a-b are plots demonstrating the ability of a C5 blocker to stop the progression of pathology of an inflamed joint. Figure 2a shows a comparison of mean joint inflammation (JI) index values. Data represent

in an JI index +/- standard error. Solid circles are C5 blocker treated mice (n=6). Open circles are control treated mice (n=4). Figure 2b shows a comparison of paw thickness of initially affected joints. After onset of joint inflammation, control paw thickness increased significantly compared to treated or normal (N) paws. Data represent mean thickness +/- SE. Solid circles are C5 blocker treated mice (n=8). Open circles are control treated mice (n=4).

Figures 3a-e are plots of paw thickness versus time for C5 blocker treated and control treated mice. Figures 3a-d show measurements from initially affected paws of paired animals; Figure 3e shows measurements from initially affected paws of two unpaired treated animals and the mean control measurements of Figure 2b.

Figures 4a-c are photomicrographs of joints stained with Hematoxylin and Eosin illustrating the use of a C5 blocker to prevent the onset of joint inflammation. Figure 4a shows a paw joint from a normal mouse; Figure 4b shows an affected paw joint from a control treated mouse, where inflammatory cell infiltration, thickening of the synovial membrane and bone erosion by the expanding synovial pannus are visible; and Figure 4c shows a typical paw joint from a C5 blocker treated mouse showing only subclinical thickening of the synovial membrane.

Figure 5 shows that treatment with a C5 blocker prevents joint inflammation. Figure 5a is a comparison of the incidence of Collagen-Induced joint inflammation in C5 blocker treated mice (n=9) and in a pool of mice treated with two different control treatments (n=10). The data represent the overall (total) incidence of joint inflammation seen within two months after the initiation of treatment. Figure 5b is a comparison of serum hemolytic activity of C5 blocker treated mice (n=5) and control treated mice (n=3) two weeks after the initiation of treatment.

Figure 6 shows the effects of C5 blocker treatment on collagen-specific humoral and cellular responses. Figure 6a was prepared by analyzing serum samples obtained from C5 blocker treated (n=4) or control treated mice (n=4) at the indicated time after CII immunization. The samples were analyzed for anti B-CII IgG in an ELISA assay. The anti B-CII antibody index represents the ratio of optical densities obtained from positive serum and negative sera. Figure 6b was prepared by culturing lymph node T cells (5×10^5) in vitro with 20 μ g/ml B-CII, C-CII, B-CI or medium only, in the presence of 5×10^5 of mitomycin C treated syngeneic spleen cells for a total of 4 days, following which the cells were pulsed with 3 H-thymidine, 18 hours before harvest.

Figure 7 shows the results of hemolytic assays demonstrating inhibition of complement activity associated with human blood circulated through an extracorporeal circuit following treatment with a C5 blocker. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 4. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Figure 8 shows the results of assays of levels of complement component C3a demonstrating that the generation of complement component C3a in whole human blood circulated through an extracorporeal circuit is not inhibited by the addition of a C5 blocker to such whole blood. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 5. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Figure 9 shows the results of assays of the levels of soluble C5b-9 (sC5b-9) in human blood circulated through an extracorporeal circuit demonstrating that the addition of a C5 blocker to such whole blood inhibits the formation of the C5b-9 terminal complement assembly. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 6. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Figures 10a-b show pharmacokinetic analyses of the reduction of the cell lysis ability of mouse blood (Fig. 10a) or extracorporeally circulating human blood (Fig. 10b) after treatment with various C5 blockers. C5 Blocker B is monoclonal antibody BB5.1; C5 Blocker F is an Fab' fragment of monoclonal antibody BB5.1; C5 Blocker N is monoclonal antibody N19-8.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to a method for treating established joint inflammation by the administration of a C5 blocker or a combination of C5 blockers to a patient in need of such treatment. As used herein, "established joint inflammation" means that the patient has at least one inflamed joint at the time treatment is commenced.

Such C5 blockers comprise proteins (including antibodies), peptides, and other molecules that directly interact with C5, C5a, and/or C5b, so as to inhibit the formation of and/or physiologic function of C5a and/or C5b. Examples of non-protein molecules of this type include K-76 COOH, (see Hong et al., 1979), and substituted dihydrobenzofurans, spirobenzofuran-2(3H)-cycloalkanes, and their open chain intermediates, (see Sindelar et al., U.S. patent No. 5,173,499), that are reported to directly interact with C5, C5a, and/or C5b. Preferably, the C5 blocker or blockers inhibit the

formation of and/or physiologic function of both C5a and C5b.

The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, 1971; Jose et al., 1990; Wurzner et al., 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used. Using assays of these or other suitable types, candidate C5 blockers, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the invention and 2) determine the appropriate dosage levels of such compounds. Examples 2, 4, and 6-8 illustrate the use of hemolytic and soluble C5b-9 assays with C5 blockers comprising monoclonal antibodies.

Blockers affecting C5a are preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25%) in the C5a levels present in at least one blood-derived fluid of the patient, e.g., blood, plasma, or serum, following activation of complement within the fluid. Alternatively, they are used at concentrations providing at least about a 10% reduction in the C5a levels present in the synovial fluid of an inflamed joint.

Similarly, blockers affecting C5b are preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25%) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. Alternatively, they are used at concentrations providing at least about a 10% reduction in the C5b levels present in the synovial fluid of an inflamed joint. In the case of C5b, such concentrations can be conveniently determined by measuring the cell-lysing

ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid (see, for example, Example 6 below). Accordingly, a preferred concentration for a C5 blocker 5 that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement 10 present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer, 1961, pages 135-139, or a conventional variation of that assay such as the chicken 15 erythrocyte hemolysis method described below.

Preferred C5 blockers are relatively specific, and do not block the functions of early complement components. In particular, such preferred agents will not substantially impair the opsonization functions 20 associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 25 convertases, and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, preferred C5 blockers do not substantially interfere with the cleavage 30 of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios by the actions of the C3 convertases. Such measurements are informative because C3a and C3b 35 levels will be reduced (compared to a matched sample without the C5 blocker) if cleavage is interfered with by a C5 blocker.

In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid 5 phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g., that sold by Quidel Corporation, San Diego, CA, according to the 10 manufacturers specifications. Particularly preferred C5 blockers produce essentially no reduction in body fluid C3a levels following complement activation when tested in such assays.

Preferred C5 blocking agents include antibodies. 15 The antibodies are preferably monoclonal, although polyclonal antibodies, which can be produced and screened by conventional techniques, can also be used if desired. Hybridomas producing monoclonal antibodies (mAbs) reactive with complement component C5 can be obtained 20 using complement component C5, C5a, and/or C5b, preferably in purified form, as the immunogen.

The most preferred antibodies will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with 25 C5a and preventing the assembly of the membrane attack complex associated with C5b. As discussed above, in a particularly preferred embodiment, these C5 blocking antibodies will not impair the opsonization function associated with the action of C3b.

General methods for the immunization of animals (in 30 this case with C5, C5a, and/or C5b), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of 35 hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case the immunogen or a molecule containing the

immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., Cellular Immunol. 127:337-351, 1990; Wurzner, et al., Complement Inflamm. 8:328-340, 1991; and Mollnes, et al., Scand. J. Immunol. 28:307-312, 1988.

A description of the preparation of a mouse anti-human-C5 monoclonal antibody with preferred binding characteristics is presented below in Example 8. Wurzner, et al., 1991, describe the preparation of other suitable mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

As used herein, the terms "antibody" or "antibodies" refer to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g., Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed (engineered) antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, the term "monoclonal" refers to any antibody that is not of polyclonal origin.

Publications describing methods for the preparation of engineered antibodies, in addition to those listed immediately above, include: Reichmann, et al., Nature, 332:323-327, 1988; Winter and Milstein, Nature, 349:293-299, 1991; Clackson, et al., Nature, 352:624-628, 1991;

Morrison, Annu Rev Immunol, 10:239-265, 1992; Haber, Immunol Rev, 130:189-212, 1992; and Rodrigues, et al., J Immunol, 151:6954-6961, 1993. Human or humanized antibodies are preferred for administration to human patients.

To achieve the desired reductions of body fluid parameters, such anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

Dosage levels of the antibodies for human subjects are generally between about 0.5 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 1 mg per kg and about 50 mg per kg per patient per treatment. In terms of body fluid concentrations, the antibody concentrations are preferably in the range from about 5 μ g/ml to about 500 μ g/ml.

Other C5 blockers can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular C5 blocker being administered.

Doses of C5 blockers will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the

monitoring of clinical endpoints such as number of joints involved, redness of joints, swelling of joints, mobility of joints, pain levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical

5 outcome.

The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the C5 blocker, and the levels of the blocker in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the C5 blocker in the body fluid may be monitored during the course of treatment.

Alternatively, for C5 blockers that affect C5b, levels of the cell-lysing ability of complement present in one or more of the patient's body fluids are monitored to determine if additional doses or higher or lower dosage levels are needed. Such doses are administered as required to maintain at least about a 25% reduction, and preferably about an 50% or greater reduction of the cell-lysing ability of complement present in blood, plasma, or serum, or at least about a 10% reduction of the cell-lysing ability of complement present in synovial fluid from an inflamed joint. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the C5 blocker or blockers used in the practice of the invention means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment. In yet another alternative, dosage parameters are adjusted as needed to achieve a substantial reduction of C5a levels in blood, plasma, or serum, or at least a 10% reduction of the C5a levels in the synovial fluid of an inflamed joint. As discussed above, C5a levels can be measured using the techniques

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described in Wurzner, et al., Complement Inflamm 8:328-340, 1991. Other protocols of administration can, of course, be used if desired as determined by the physician.

5 In a preferred embodiment, administration of the C5 blocker is initiated when the patient experiences a "flare up" of joint inflammation in which one or more affected joints becomes more swollen and takes on an erythematous (reddened) appearance.

10 Administration of the C5 blockers will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used 15 if desired and practicable for the particular C5 blocker to be administered.

20 For the treatment of established joint inflammation by systemic administration of a C5 blocker (as opposed to local administration, e.g., intra-articular injection into the inflamed joint) administration of a large initial dose is preferred, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large 25 initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

30 Formulations suitable for injection, p.o., and other routes of administration are well known in the art and may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). Parenteral formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline,

5 buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. These formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

10 The formulations of the invention can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the C5 blocker or blockers and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material will include a label which indicates that the formulation is for use in the treatment of joint inflammation and may specifically refer to arthritis, rheumatoid arthritis, osteoarthritis, lupus arthritis, 15 psoriatic arthritis, juvenile onset rheumatoid arthritis, reactive arthritis, Reiter's syndrome (Reiter's disease), or other diseases involving joint inflammation.

20 Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

MATERIALS AND METHODS

Cell Lysis Assays

25 The cell-lysing ability of complement in various body fluid samples was determined using hemolytic assays performed as follows: Chicken erythrocytes were washed well in GVBS (Sigma Chemical Co. St. Louis, MO, catalog No. G-6514) and resuspended to 2×10^8 /ml in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, NJ) was added to the cells at a final concentration of 25 $\mu\text{g}/\text{ml}$ and the cells were incubated for 15 min. at 23°C. 30 The cells were washed 2x with GVBS and 5×10^6 cells were resuspended to 30 μL in GVBS. A 100 μL volume of body fluid test solution was then added to yield a final 35

reaction mixture volume of 130 μ L. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent of a body fluid (including serum, as well as other body fluids such as 5 blood, plasma, or synovial fluid) in the 100 μ L volume of body fluid test solution.

After incubation for 30 min. at 37°C, percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the 10 cells down and measuring released hemoglobin in the supernatant as the optical density at 415nm.

A 50% reduction in hemolysis after treatment with the C5 blocker or blockers used in the practice of the invention means that the percent hemolysis after treatment was one half of the percent hemolysis before treatment.

Various hemolytic assays described below in the examples were performed using this chicken erythrocyte assay with the following body fluid inputs. For assays 20 of mouse complement activity, the 100 μ L volume of body fluid test solution contained 50 μ L of diluted (in GVBS) mouse serum and 50 μ L of human C5 deficient serum (Quidel Corporation, San Diego, CA). For assays of human complement activity, the body fluid test solution 25 contained various concentrations of human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the final 100 μ L volume. For the assays used to screen hybridoma supernatants discussed below in example 8 each 100 μ L volume of serum test solution 30 contained 50 μ L of hybridoma supernatant and 50 μ L of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

Collagen Induced Joint Inflammation

Examples 1, 2, and 3 use a collagen induced joint 35 inflammation system that has been employed since the 1970s as an animal model of human joint inflammation, particularly RA (see, for example, Trentham et al., 1977;

Holmdahl et al., 1986; Boissier et al., 1987; Yoo et al., 1988). This model system was implemented using 8-12 week old male DBA/1LacJ mice that were purchased from The Jackson Laboratory (Bar Harbor, ME).

5 Immunization

Bovine collagen II (B-CII) obtained from Elastin Products Company, Inc., Owensville, MO, was dissolved in 0.01M acetic acid by stirring overnight at 4°C at a concentration of 4mg/ml. Complete Freund's adjuvant (CFA) was prepared by the addition of desiccated Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) to incomplete Freunds adjuvant (Difco) at a concentration of 2mg/ml. The solution of B-CII was emulsified in an equal volume of CFA and a 100 μ l aliquot of this emulsion, containing 200 μ g B-CII and 100 μ g of Mycobacterium, was injected intradermally at the base of the mouse's tail. After 21 days, all mice were reimmunized using the identical protocol. This secondary CII reimmunization served primarily to boost the serum levels of anti-CII antibodies in the immunized mice. After CII reimmunization, the onset of joint inflammation (JI) and disease progression rise dramatically, characterized by the severe swelling and redness of the joints of one or more paws at around 4-6 weeks after the initial immunization.

25 Clinical evaluation

Mice were examined daily beginning on the day of reimmunization for the appearance of JI. The presence of JI was determined by examining, measuring and scoring each of the forepaws and hindpaws. Collagen induced JI (CIJI) is characterized by swelling and erythema or visible joint distortion of one or more extremities. The severity of JI in each affected paw was scored as: 0 - normal joint; 1 - visible redness and swelling; 2 - severe redness and swelling affecting entire paw or joint; or 3 - deformed paw or joint with ankylosis. The sum of the scores for all four paws in each mouse was

used as an index (the "JI index") to assess overall disease severity and progression.

Anti-complement Monoclonal Antibodies

Monoclonal antibodies that bind to and block mouse C5 were prepared by standard methods from hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research, Mill Hill, London, England. Anti-human C8 hybridoma, 135.8, which generates an Mab that does not block mouse C8, was obtained from Dr. Peter Sims (Blood Research Institute, Milwaukee, WI). Both antibodies are IgG1 isotypes, and ascites of BB5.1 or 135.8 were obtained in athymic nude mice or BALB/c mice, respectively. IgGs were purified from ascites with a protein A affinity column eluted with acetic acid, and subsequently dialyzed against PBS. Purified antibodies were quantified by spectrophotometric determination of absorbance at 280nm and sterilized with a 0.22 μm filter.

Antibody administration

For prophylactic treatment, mice were randomly divided as C5 blocker treated and control treated groups and subsequently received 750 μg per mouse ip doses of either anti-mouse C5 mAb, BB5.1, or anti-human C8 mAb, 135.8, as a control, twice weekly. For therapeutic treatment of established JI, mice received anti-mouse C5 mAb BB5.1 or anti-human C8 mAb 135.8 at 2-5 mg/mouse ip daily for 10 days after the initial onset of JI was observed. The doses of anti-C5 mAb were adjusted in a range spanning 2 mg to 5 mg per injection to ensure that the desired depletion of C5 mediated hemolytic activity was obtained, i.e., a depletion of at least 50%.

Unlike the situation in humans, where administration of a C5 blocker to a body fluid of genetically unrelated individuals results in roughly equivalent levels of complement inhibition, the dose of anti-murine-C5 mAb required to deplete hemolytic activity by a given amount in mice is strain dependent. The dose required to

deplete hemolytic activity in DBA/1LacJ mice is approximately four times higher than the dose required to achieve an equivalent depletion of the hemolytic activity in BALB/c mice.

5 T cell stimulation assays

Lymph node cells taken from animals at the time of sacrifice were analyzed for specific T cell responses to collagen II. T cells (5×10^5) were incubated with 5×10^5 mitomycin C (50 μ g/ml) treated syngeneic spleen cells from normal DBA/1LacJ mice in flat bottomed 96-well plates. Bovine collagen II (B-CII), bovine CI (B-CI), chicken CII (C-CII) and ovalbumin or BSA were added to cultures at 20 μ g/ml. The culture medium was RPMI-1640 supplemented with 5% heated inactivated FCS, 5×10^{-5} M 2-ME, 10mM HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, and 1% penstrep. The cultures were incubated at 37°C in 5% CO₂ for 4 days. Eighteen hours before harvesting, 1 μ ci of ³H-thymidine was added to each well. Results are expressed as cpm obtained from triplicate T cell cultures.

20 Quantification of anti B-CII antibodies

Mice were bled at various times after immunization with B-CII. Serum anti B-CII antibody titers were measured using a conventional ELISA for B-CII similar to the ELISA for anti C5 antibodies described below in Example 8, but using B-CII to coat the plates (also see Myers, et al., 1989, and Seki et al., 1992, for descriptions of similar assays).

25 Histological examination

30 Mice from each group were sacrificed and all four legs from each mouse were fixed in 10% buffered formalin and decalcified in a solution of 3.1% HCL, 5% formic acid and 7% aluminum chloride. The tissue samples were embedded in paraffin, sectioned at 5 μ m and stained with hematoxylin and eosin. For immunofluorescence staining, paws were decalcified in a 0.1M Tris solution containing 10% EDTA and 7.5% PVP for 3 days and frozen in OCT at

-80°C. 5µm sections were then prepared and stained with 9FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, CA, Catalog No. 65-6411) at a dilution of 1 to 50.

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EXAMPLE 1Therapeutic Effects of C5 Blocker Treatment after the Clinical Onset of Collagen Induced Joint Inflammation

In order to assess the effects of administration of a C5 blocker on established JI, mice were observed following induction of CIJI, as described above, and pairs of mice were selected that showed the initial appearance of readily detectable JI symptoms (swollen joints of the paws) on the same day. One mouse of each such pair was treated with a anti-C5 mAb BB5.1 and one was treated with a control injection of either the irrelevant mAb 135.8 or PBS. In each matched pair, the animal with the greatest overall level of paw inflammation was assigned to the C5 blocker treatment group so as to avoid potentially biasing the results in favor of the C5 blocker treatment. Starting on the first day when joint inflammation was observed as paw inflammation, treatments were continued daily for 10 days. (In one case a pair of mice was only carried for 8 days.) In addition to these matched pairs, two unpaired animals also received the C5 blocker treatment after the induction of JI.

Histological examination of initially affected joints from control-treated mice at the end of the treatment period revealed extensive bone erosion with severe inflammatory cell infiltration, thickening of the synovial membranes, and pannus formation (Figure 1b). In contrast, the initially affected joints of the C5 blocker treated group showed preserved joint structure with some degree of thickening of synovial membranes and mononuclear cell infiltration into some of the joints (Figure 1c). The severe inflammatory cell infiltration in the control-treated joints was predominantly made up

of polymorphonuclear cells (PMNs, neutrophils). Surprisingly, such PMN infiltration was almost completely absent in the C5 blocker treated mice.

During the clinical course of CIJI, an important indicator of the progression of disease is the involvement of additional limbs. Therefore, the number of limbs with clinically detectable JI at the end of the treatment period was compared with the number of limbs exhibiting JI symptoms before the start of therapy. The severity and progression of JI in each affected paw was determined and scored as described above under the heading "Materials and Methods", and the sum of the scores for all four paws of each animal was used as a "JI index". The thicknesses of all four paws of each animal were also measured with a caliper during the time of this experiment to provide a completely objective evaluation of this aspect of disease progression.

As shown in Table 1 (mean values) and Table 2 (individual values), there were significant increases in new limb involvement in the control treated group during the course of 10 day treatment, while the number of inflamed limbs was decreased when DBA/1LacJ mice with inflamed joints were treated with the C5 blocker starting at the time of disease onset. In addition to new limb recruitment, the initially affected paws of the control treated animals evidenced progression of inflammatory joint disease severity by becoming more inflamed (Figure 2a). Acute inflammation in the affected joints was observed as severe joint swelling and redness during the first few days, followed by joint deformation and ankylosis at the end of 10 day period. In contrast, no new paws were involved and the severity of inflammation in the majority of affected joints subsided or remained unchanged during the course of C5 blocker therapy (Figure 2a).

The paw thicknesses of initially affected limbs in both C5 blocker treated and control treated groups during

the course of these experiments is shown as mean values for each group in Figure 2b. Figures 3a, 3b, 3c, and 3d show values for each initially inflamed paw of each of the matched pairs of control treated and C5 blocker treated animals, while Figure 3e shows the values obtained for each initially inflamed paw of each of the unpaired C5 blocker treated animals (shown along with the mean values for control treated animals of Figure 2b). In these figures, the number in parenthesis indicates the designation of the particular animal, while the letters following the numbers (only in those cases where more than one limb was affected initially) indicate the particular paws affected, with the first letter indicating front (F) or rear (R) paws, and the second letter indicating right (R) or left (L) paws.

As can be seen in Figures 2 and 3, and in Tables 1 and 2, C5 blocker treatment successfully prevented further paw recruitment and reduced (but did not completely abolish) the inflammation in the initially affected joints in all but one (mouse #4) of the C5 blocker treated animals. As can be seen in Figure 2b, the mean thickness of initially affected paws in the control treated group increased significantly during the 10 day period, while the mean thickness of initially affected paws in the C5 blocker treated group decreased, but not significantly.

EXAMPLE 2

Prophylactic Treatment with a C5 Blocker Prevents Collagen Induced Joint Inflammation

In these experiments, the administration of the C5 blocker coincided with the reimmunization of the experimental animals with B-CII. On the day of reimmunization, mice were symptom free, and were randomly assigned to C5 blocker treatment or control treatment groups. Each mouse was treated with either the C5 blocker (anti-mouse C5 mAb, BB5.1) or a control treatment (anti-human C8 mAb, 135.8) at 750 μ g per mouse ip twice

weekly. The animals were treated for four weeks, at which time treatment was discontinued. The results of this study are shown in Figures 4 and 5.

Administration the C5 blocker completely prevented the development of CIJI (0/8). All mice in the C5 blocker treated group exhibited no signs of clinical disease during the period of treatment (and for up to two months after discontinuing the C5 blocker therapy in the two animals followed for that long). In contrast, 90% of the control treated animals (9/10) developed JI by 4-6 weeks after the first B-CII immunization. The percent incidence of JI observed in the control treated and C5 blocker treated animals after 4-6 weeks is plotted in Figure 5a. (Note that the value for the C5 blocker treated group in this figure is actually 0%, but a bar indicating 1% has been plotted in order to indicate that the data for this set of animals was obtained and is presented.) Peak inflammation levels were observed around 5 weeks after the initial collagen immunization. As shown in Figure 5b, 80% to 90% of the serum hemolytic activity was depleted in the C5 blocker treated group, while the serum hemolytic activity remained normal in the control treated group.

As shown in Figure 4, histological examination of affected joints from control mice revealed extensive mononuclear cell as well as polymorphonuclear cell infiltration, thickening of the synovial membrane and bone erosion by the expanding synovial pannus (Figure 4b). In contrast, there were no signs of inflammatory processes observed in the majority of joints studied from the C5 blocker treated mice. A few joints from these C5 blocker treated mice showed some subclinical thickening of the synovial membrane, but this alteration was not accompanied by any visible bone erosion or inflammatory cell infiltration (Figure 4c). Interestingly, immunofluorescence staining showed antibody deposition along cartilage surfaces and C3 activation at synovial

membranes in the joints of both the control treated and the C5 blocker treated animals.

EXAMPLE 3

5 Effect of C5 Blocker Treatment on the Humoral and Cellular Immune Responses to Immunization with Collagen

Responses of both the humoral and cellular immune systems are activated after immunization of DBA/1LacJ mice with bovine Collagen II. Anti B-CII titers increase, and the serum IgG anti B-CII titers in C5 10 blocker treated mice are equivalent to those of control treated mice when tested at 14, 28 and 42 days after the initial B-CII immunization (Figure 6a). Anti B-CII antibody titers from both control and anti-C5 mAb treated mice rise significantly after the B-CII reimmunization 15 and remain at the resulting plateau for an extended period of time.

In order to study T cell responses, lymph node cells (LNCs) from C5 blocker treated mice and control treated mice were cultured with either B-CII, B-CI, C-CII, or 20 culture medium only. LNCs from either C5 blocker treated mice or control treated mice responded specifically and equally to B-CII regardless of the treatment the animals received concurrently with B-CII reimmunization. C-CII, which shares many conserved regions of homology with B- 25 CII also elicited a moderate T cell response when cultured with LNCs from C5 blocker treated mice or from control treated mice. In contrast, LNCs from age matched non-immunized mice responded poorly to all of the tested collagens (Figure 6b).

30 The data obtained in these experiments and those of Examples 1 and 2 clearly demonstrate that in vivo administration of a C5 blocker prevents the development and progression of CIJI and that this treatment does not interfere with the humoral and cellular immune responses 35 seen after immunizing mice with bovine type II collagen. Both collagen-specific T cell responses and anti-CII antibody titers were comparable in both the C5 blocker

treated mice and the control treated B-CII reimmunized mice.

EXAMPLE 4

C5 Blocker Inhibition of Complement Activity

5 The effects of a C5 blocker on complement activation were evaluated using a closed-loop cardio-pulmonary bypass (CPB) model for the extracorporeal circulation of human blood. As discussed fully in copending U.S. patent application Serial No. 08/217,391, filed March 23, 1994, 10 extracorporeal circulation of human blood causes activation of complement in the blood.

The C5 blocker was a monoclonal antibody raised in mice against purified human C5 protein (Wurzner, et al., Complement Inflamm 8:328-340, 1991; mAb N19-8) that was 15 propagated, recovered and purified as an IgG fraction from mouse ascites fluid (Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Current Protocols In Immunology, John Wiley & Sons, New York, 1992).

20 To carry out these experiments, 300 ml of whole human blood was drawn from a healthy human donor and additionally a 1 ml sample was removed as a control sample for later analysis. The blood was diluted to 600 ml by the addition of Ringer's lactate solution containing 10U/ml heparin. The C5 blocker (30 mg in 25 sterile PBS) was added to the diluted blood to a final concentration of 50 µg/ml. In a control experiment, an equal volume of sterile PBS was added to the diluted blood. The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator CPB machine (Cobe BCT, Inc., Lakewood, CO) and the circuit was started. The circuit was cooled to 28°C 30 and circulated for 60 minutes. The circuit was then warmed to 37°C and circulated for an additional 30 minutes. The experiment was then terminated. Samples 35 were taken at several time points and evaluated for complement activity (Figure 7).

At each time point an aliquot of whole blood was taken, divided into 3 samples and A) diluted 1:1 in 2% paraformaldehyde in PBS to evaluate platelet and blood cell activation parameters as discussed in the above-referenced U.S. patent application Serial No. 08/217,391; B) centrifuged to remove all cells and plasma diluted 1:1 in Quidel sample preservation solution (Quidel Corporation, San Diego, CA) and stored at -80°C, following which these frozen diluted plasma samples were thawed and used to evaluate C3a and C5b-9 generation (Examples 5 and 6, respectively), and C) centrifuged to remove all cells and undiluted plasma stored at -80°C, following which these frozen plasma samples were thawed and hemolytic assays were performed as described above.

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As can be seen in Figure 7, addition of the C5 blocker to the extracorporeal circuit resulted in a 95% reduction of the cell-lysing ability of complement in the plasma. The complement activity remained inhibited throughout the course (90 minutes) of the experiment.

EXAMPLE 5

Generation of C3a in the Presence of a C5 Blocker

The fresh frozen plasma samples that had previously been diluted in Quidel sample preservation solution following CPB circulation (see Example 4) were assayed for the presence of the complement split product C3a by using the Quidel C3a EIA kit (Quidel Corporation, San Diego, CA). These measurements were carried out according to the manufacturer's specifications. C3a released is expressed in ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in Figure 8, addition of the C5 blocker had no effect on the production of C3a during the CPB experiment. C3a generation was dramatically increased during the final 30 min. of the experiment and correlates with sample warming.

EXAMPLE 6Prevention of the Generation of C5b-9 by a C5 Blocker

Fresh frozen plasma samples that had been previously diluted in Quidel sample preservation solution following 5 CPB circulation (see Example 4) were assayed for the presence of the terminal human complement complex C5b-9 using the Quidel C5b-9 kit (Quidel Corporation, San Diego, CA). The amount of soluble C5b-9 (sC5b-9) in each 10 sample was determined using the manufacturers specifications and is expressed in arbitrary absorbance units (AU).

As can be seen in Figure 9, the C5 blocker completely inhibited C5b-9 generation during extracorporeal circulation so that sC5b-9 levels during 15 the full course of the run were equivalent to control (t_0) time points. The results of this experiment and those of Examples 4 and 5 show that the addition of a C5 blocker to human blood undergoing extracorporeal circulation effectively inhibits both complement hemolytic activity (Figure 7) and C5b-9 generation 20 (Figure 9), but not C3a generation (Figure 8).

Example 7Pharmacokinetics of mAb C5 Blockers

The *in vivo* duration of action of mAb BB5.1, and a 25 Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, ME. The mice were given 30 a single intravenous injection (at 35 mg/kg body weight) of the mAb or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb 35 BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

Fig. 10a shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined by testing serum obtained from the blood and diluted to 2.5% in hemolytic assays, as described above) after the in vivo administration of the intact mAb, the Fab' fragment, or the PBS. As shown in the figure, the intact mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the C5 blocker treated animals appeared the same as those taken from the PBS control treated animals. The overall appearance of these test and control mice was also indistinguishable prior to necropsy.

An anti-human C5 mAb was also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis-inhibiting effects of this C5 blocker were assayed over a 90 minute period of circulation. The results of these assays are charted in Figure 10b, and show that the C5 blocker essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that these C5 blockers will survive in the bloodstream for a substantial period of time, thus making periodic administration practical.

EXAMPLE 8

Preparation of a C5 Blocker

A C5 blocker mAb suitable for use in the practice of the present invention was prepared as follows.

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, CA, Catalog #A403). The first injection contained 100 μ g of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100 μ g of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100 μ g of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100 μ g of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, MO, Catalog No. P-7181) and plated at 1-2.5 x 10⁵ cells per well. Selection in EX-CELL 300 medium (JRH Biosciences, Lexena, KS, Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, MO, Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred to EX-CELL 300 medium supplemented with FBS, GPS, and HT (Sigma Chemical Company, St. Louis, MO,

Catalog No. H-0137) for 1 week and then cultured in EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10-14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol.

A 50 μ L aliquot of a 2 μ g/ml solution of C5 (Quidel Corporation, San Diego, CA) in sodium carbonate/bicarbonate buffer, pH 9.5, was incubated overnight at 4°C in each test well of a 96 well plate (Nunc-Immuno F96 Polysorp, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 μ L of blocking solution, 1% BSA in TBS (BSA/TBS), for 1 hour at 37°C. After an additional wash step, a 50 μ L aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37°C with a subsequent wash step. As a secondary (detection) antibody, 50 μ L of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37°C, followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, MO, Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, MO, Catalog No. P-4922), and 50 μ L of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50 μ L aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in the chicken erythrocyte hemolysis assay.

5 Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize

10 serum hemolytic activity.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America, on April 27, 1994, and has been assigned the designation HB-11625.

15 This deposit were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977).

Throughout this application various publications and patent disclosures are referred to. The teachings and disclosures thereof, in their entireties, are hereby incorporated by reference into this application to more fully describe the state of the art to which the present invention pertains.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

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TABLE 1

		NUMBER OF LIMBS AFFECTED PER GROUP*			
Treatment	n	Day 0	Day 10	% Change	% Hemolytic Activity
Control	4	4 (1.0)	9 (2.3)	+125.0	95.6 ± 3.8
C5 Blocker	6	8 (1.3)	7 (1.2)	- 12.5	13.9 ± 4.7

* Numbers in parenthesis represent the average number of affected joints per mouse.

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TABLE 2

Mouse (treatment)	NUMBER OF LIMBS AFFECTED PER MOUSE			% Change	% Hemolytic Activity
	Day 0	Day 10			
#8 (control)	1 (RL)	2 (RL & RR)	+100	91.2	
#2 (C5 blocker)	1 (FL)	1 (FL) *	0	22.4	
#6 (control)	1 (RR)	2 (RR & RL)	+100	103.2	
#5 (C5 blocker)	1 (RR)	1 (RR)	0	6.0	
#1 (control)	1 (FL)	2 (FL & FR)	+100	92.4	
#4 (C5 blocker)	1 (RL)	2 (FL & RL)	+100	9.2	
#9 (control) †	1 (RL)	2 (RR & RL)	+100	not tested	
#3 (C5 blocker) †	2 (FL & RR)	1 (FL)	-100	0.4	
#7 (C5 blocker) §	1 (FR)	1 (FR)	0	13.6	
#10 (C5 blocker) §	2 (RL & RR)	1 (RR) **	-100	32.0	

* barely detectable inflammation

** only 1 toe

† data represent only 8 days for mouse #9 and #3

§ mouse #7 and #10 were not provided with matched controls

What is claimed is:

1. A method for the treatment of established joint inflammation in a human or non-human patient in need thereof comprising administering to the patient an effective anti-inflammatory amount of a C5 blocker.
2. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to substantially inhibit the cell-lysing ability of complement present in a blood-derived fluid of the patient.
3. The method of Claim 2 wherein the blood-derived fluid is serum.
4. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to substantially reduce the level of soluble C5b-9 present in a blood-derived fluid of the patient after activation of complement in that fluid.
5. The method of Claim 4 wherein the blood-derived fluid is serum.
6. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to substantially reduce the level of C5a present in a blood-derived fluid of the patient after activation of complement in that fluid.
7. The method of Claim 6 wherein the blood-derived fluid is serum.
8. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to reduce the cell-lysing ability of complement present in the synovial

fluid of an inflamed joint of the patient by at least 10%.

9. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to reduce the level of soluble C5b-9 present in the synovial fluid of an inflamed joint of the patient by at least 10%.

10. The method of Claim 1 wherein the C5 blocker is administered in an amount sufficient to reduce the level of C5a present in the synovial fluid of an inflamed joint of the patient by at least 10%.

11. The method of Claim 1 comprising the further step, after the administration of the C5 blocker, of determining the C5a level and/or the C5b level in the synovial fluid of an inflamed joint of the patient so as to monitor the course of the patient's response to the administration of the C5 blocker.

12. The method of Claim 11 wherein the C5a level is determined by an immunoassay or a chemotaxis assay.

13. The method of Claim 11 wherein the C5b level is determined by measuring the level of soluble C5b-9 in the synovial fluid or by measuring the cell-lysing ability of complement present in the synovial fluid.

14. The method of Claim 1 wherein the C5 blocker does not substantially interfere with the cleavage of complement component C3 in the patient's serum into C3a and C3b.

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15. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises a C5 blocker which provides the agent with anti-inflammatory properties; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of joint inflammation.

16. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises a C5 blocker which provides the agent with anti-inflammatory properties; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of arthritis.



Fig. 1a



Fig. 1b



Fig. 1c

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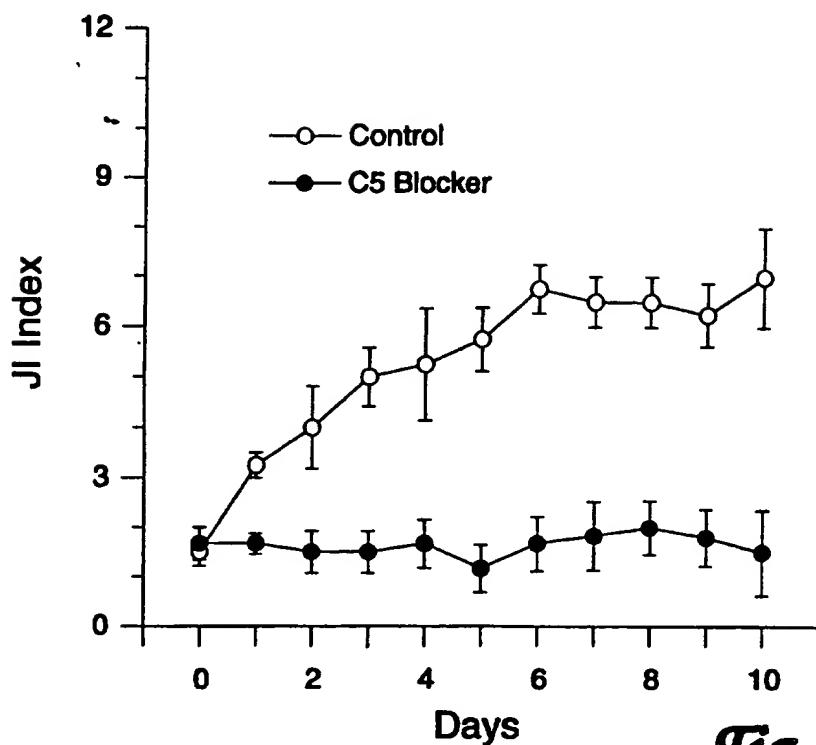


Fig. 2a

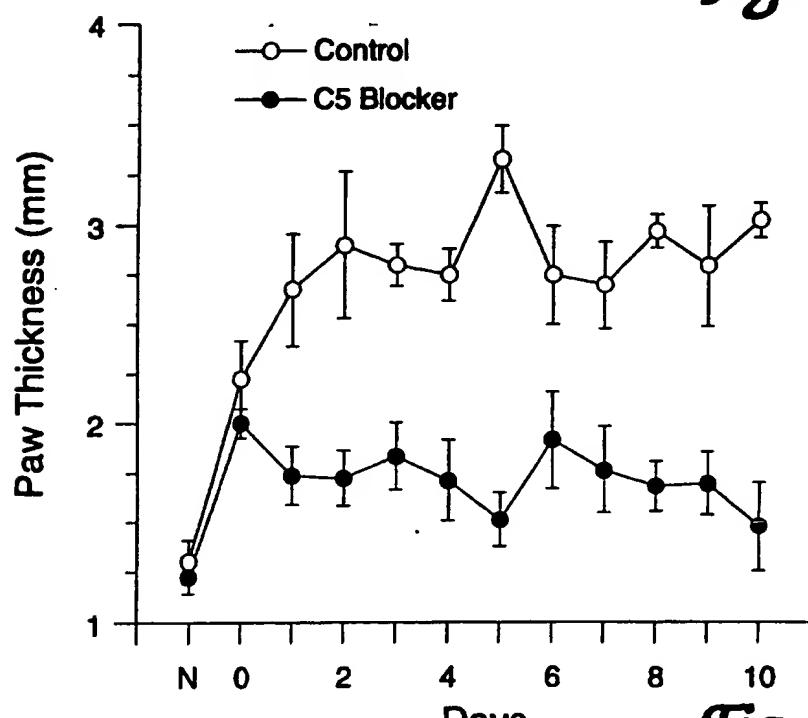


Fig. 2b

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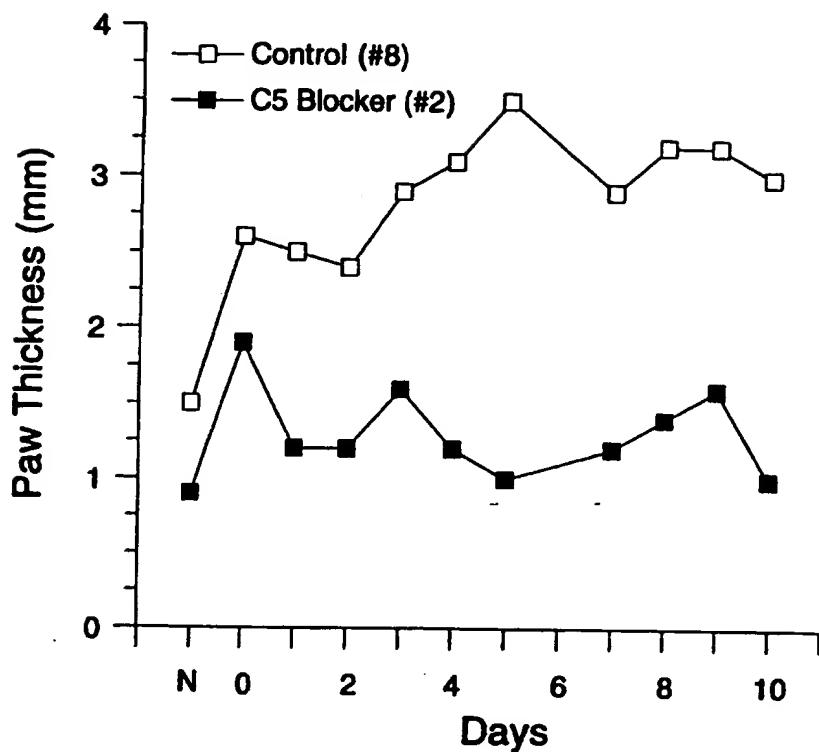


Fig. 3a

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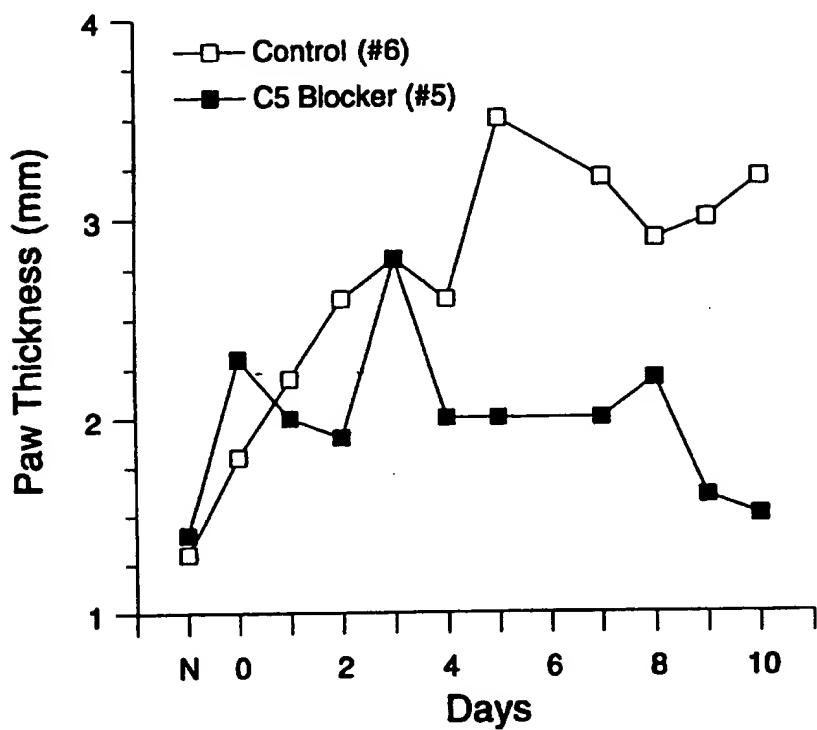


Fig. 3b

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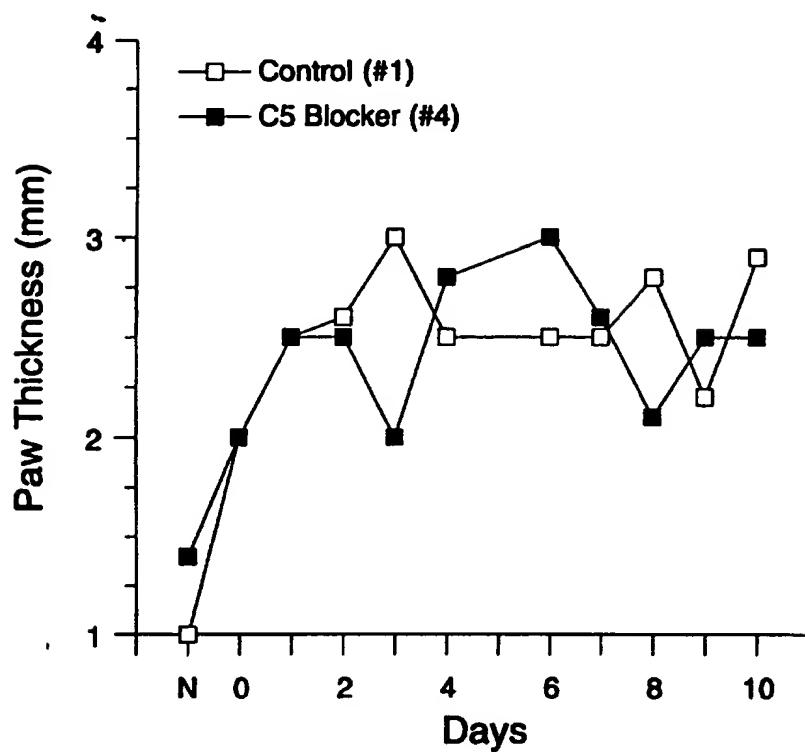


Fig. 3c

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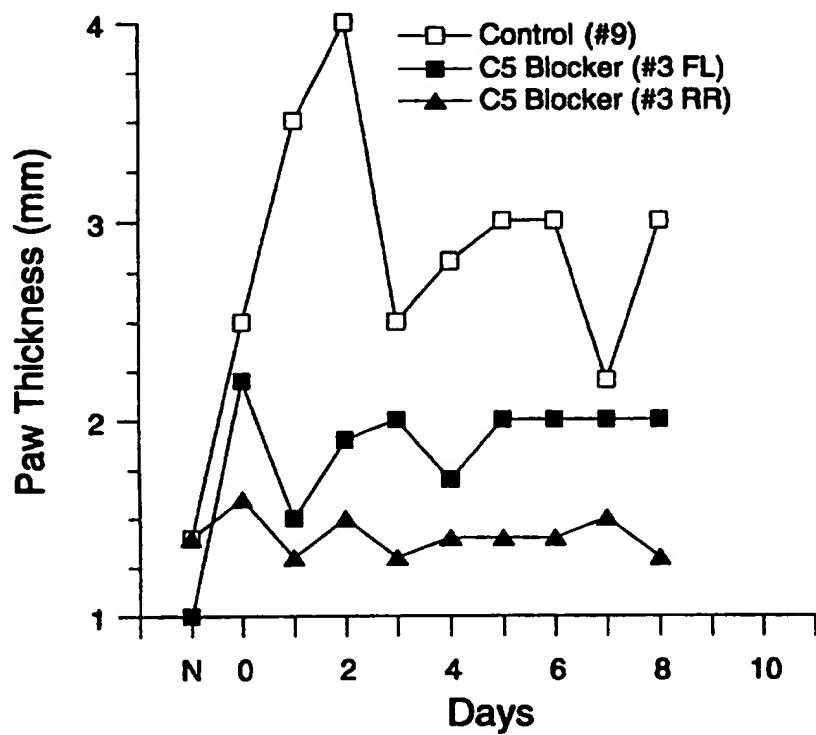


Fig. 3d

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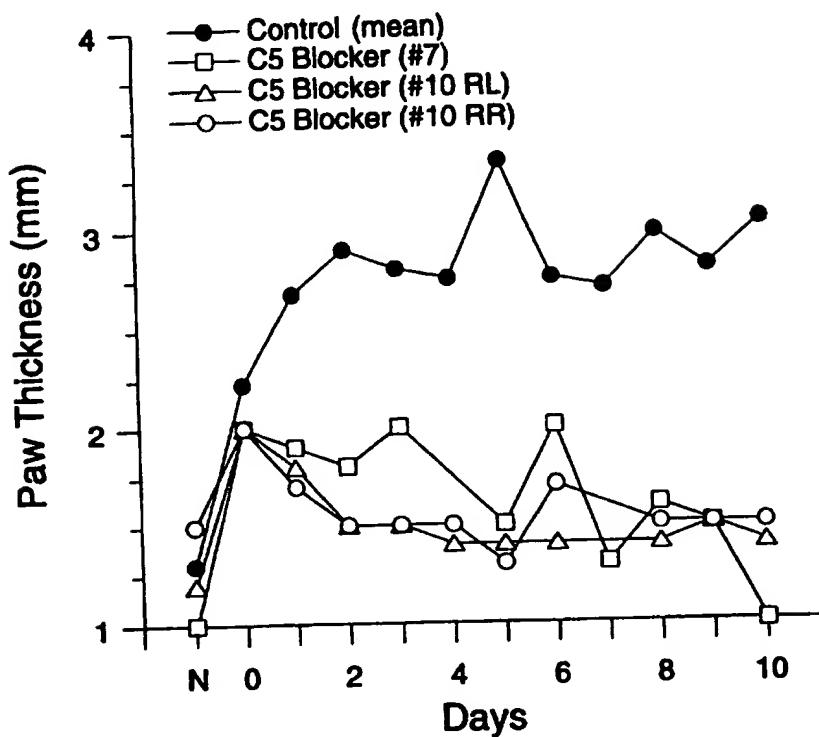


Fig. 3e

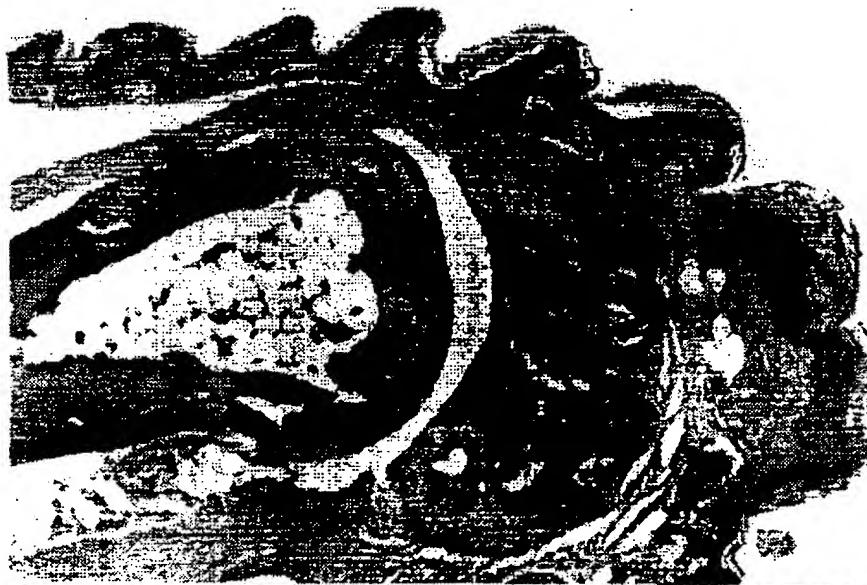


Fig. 4a



Fig. 4b



Fig. 4c

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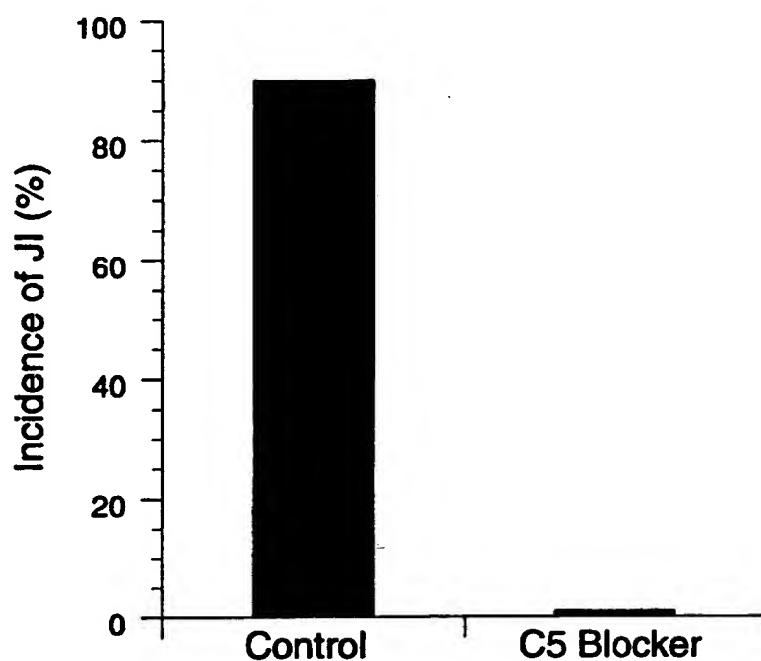


Fig. 5a

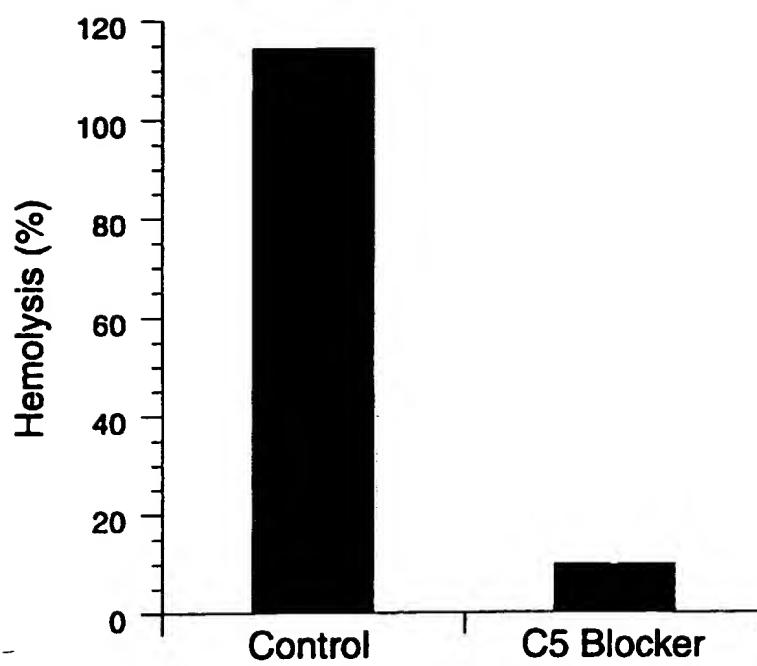


Fig. 5b

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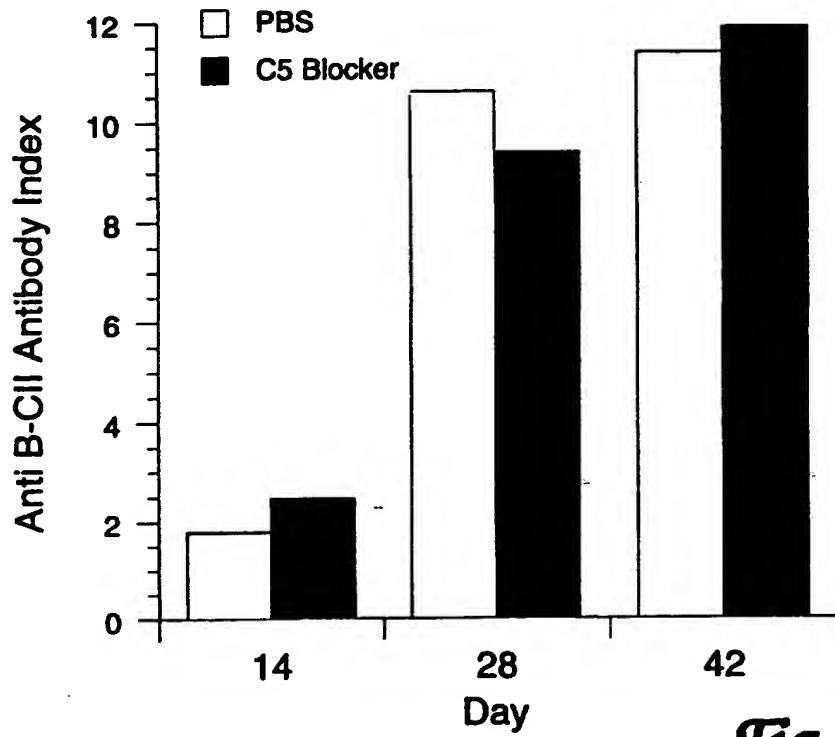


Fig. 6a

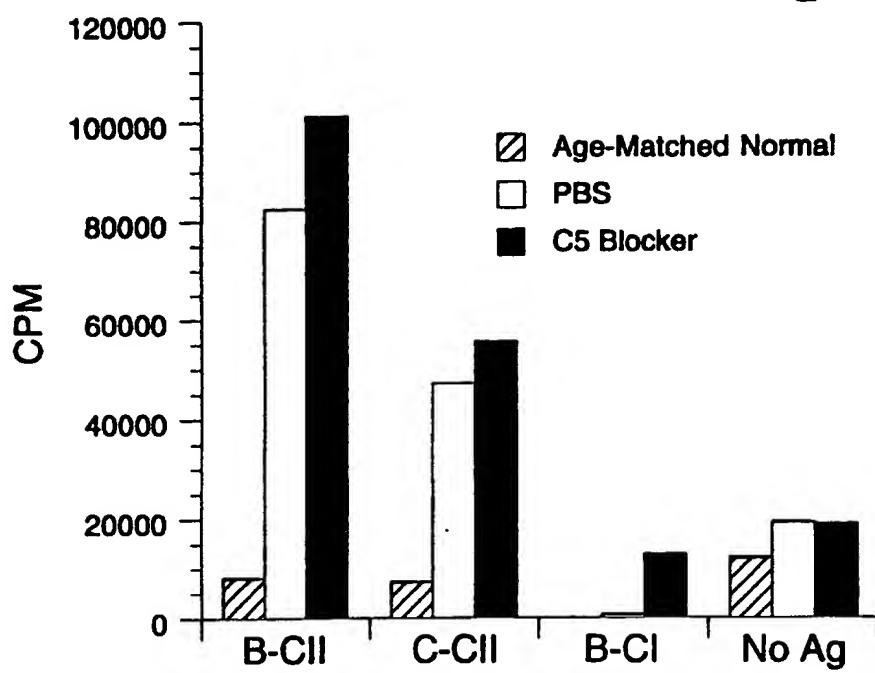


Fig 6b

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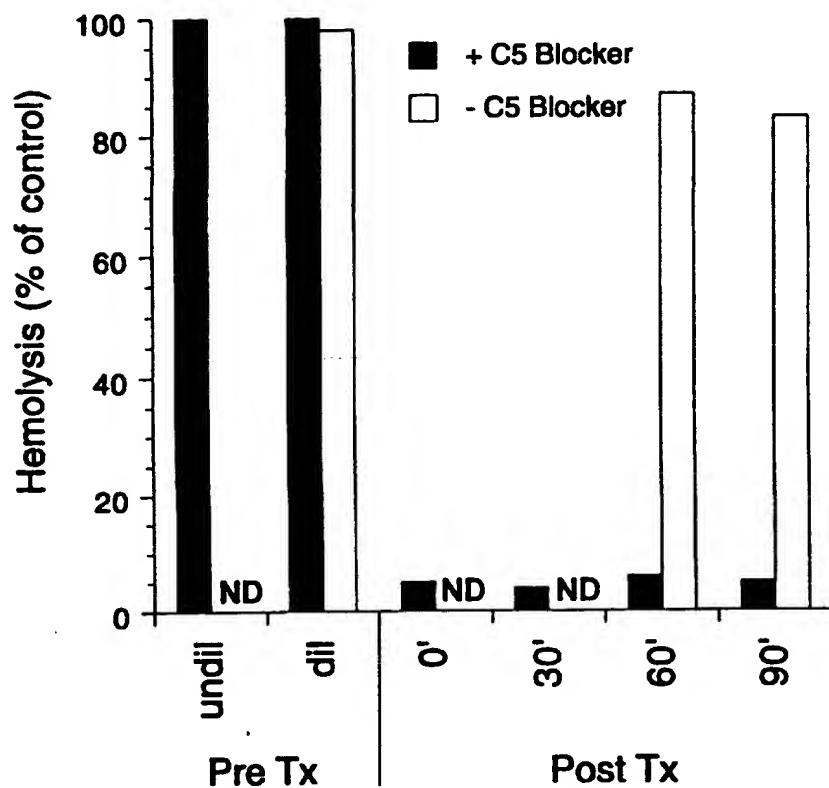
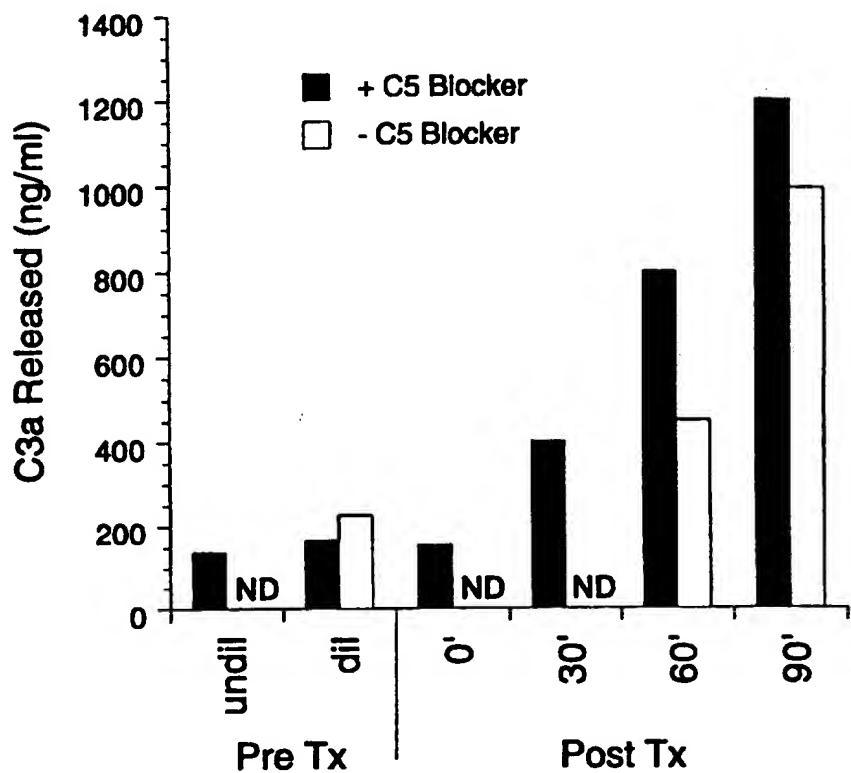


Fig. 7

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*Fig. 8*

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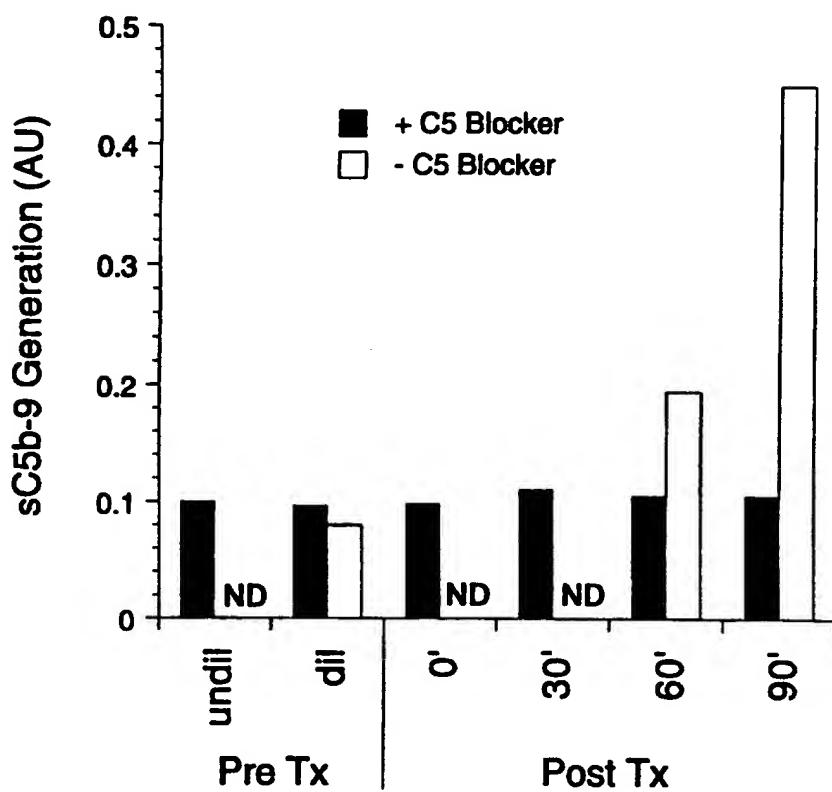


Fig. 9

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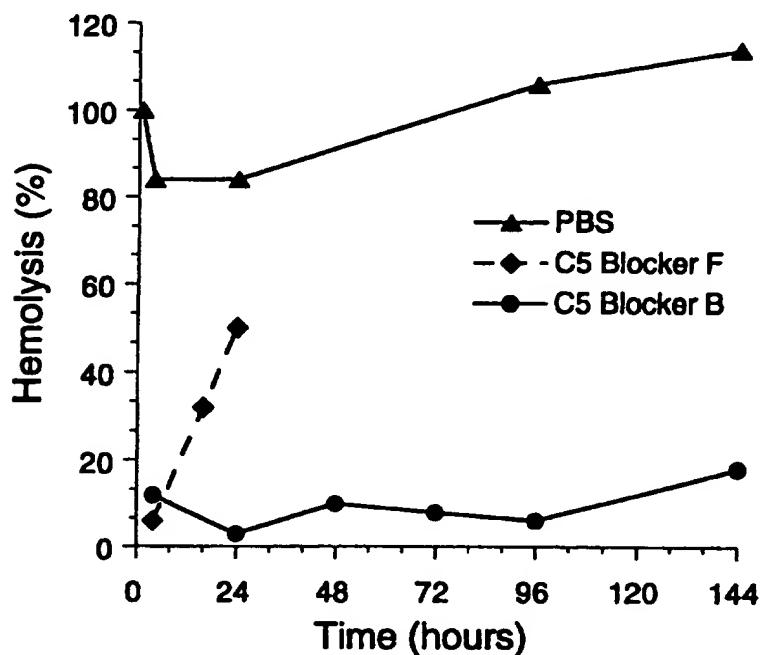


Fig. 10a

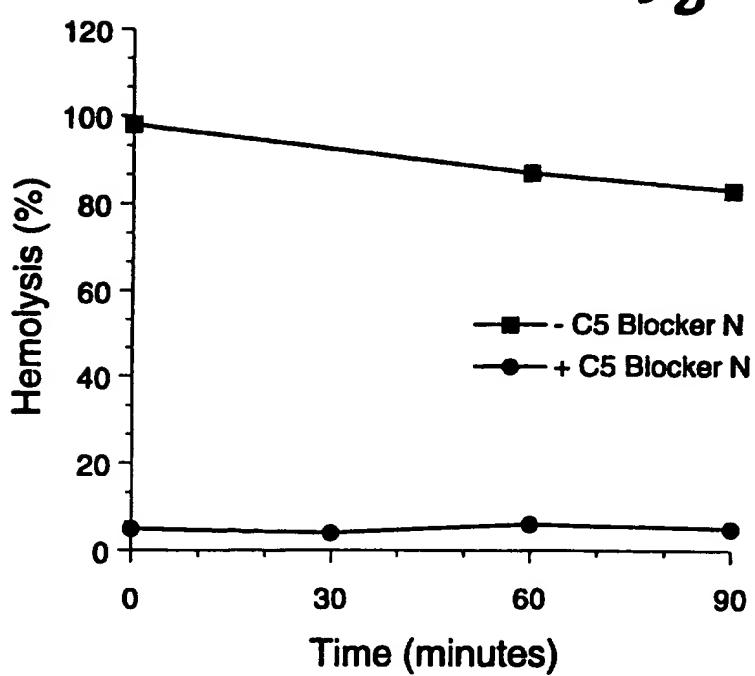


Fig. 10b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12404

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/395, 31/34; C07D 307/94; C07K 16/18, 16/40
US CL :424/130.1, 145.1; 514/462, 825; 549/345; 530/388.25, 389.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 145.1; 514/462, 825; 549/345; 530/388.25, 389.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG (EMBASE, LIFESCI, WPI, MEDLINE, BIOSYS) AND APS

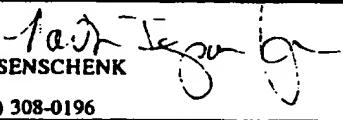
C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANNALS OF THE RHEUMATIC DISEASES, VOLUME 51, ISSUED 1992, J.J. ABBINK ET AL., "RELATIVE CONTRIBUTION OF CONTACT AND COMPLEMENT ACTIVATION TO INFLAMMATORY REACTIONS IN ARTHRITIC JOINTS", PAGES 1123-1128, SEE ENTIRE DOCUMENT.	1-16
Y	COMPLEMENT INFLAMM., VOLUME 8, ISSUED 1991, R. WURZNER ET AL., "INHIBITION OF TERMINAL COMPLEMENT COMPLEX FORMATION AND CELL LYSIS BY MONOCLONAL ANTIBODIES", PAGES 328-340, SEE ENTIRE DOCUMENT.	1-16
Y	IMMUNOGENETICS, VOLUME 35, ISSUED 1992, M. ANDERSSON ET AL., LETTER TO THE EDITOR, PAGES 71-72, SEE ENTIRE DOCUMENT.	1-16

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 NOVEMBER 1995	23 JAN 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/12404

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,173,499 (SINDELAR ET AL.) 22 DECEMBER 1992, SEE ENTIRE DOCUMENT.	1-16
Y	US, A, 5,135,916 (SIMS ET AL.) 04 AUGUST 1992, SEE ENTIRE DOCUMENT.	1-16